

Shedding of *Neospora caninum* oocysts by dogs fed tissues from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil

A.A.R. Rodrigues^a, S.M. Gennari^b, D.M. Aguiar^b,
C. Sreekumar^c, D.E. Hill^c, K.B. Miska^c,
M.C.B. Vianna^c, J.P. Dubey^{c,*}

^aDepartamento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (USP), Avenida Prof. Orlando Marques de Paiva, 87, Cidade Universitária, São Paulo, SP, CEP 05508-000, Brazil

^bDepartamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil

^cAnimal Parasitic Diseases Laboratory, United States Department of Agriculture, Animal and Natural Resources Institute, Agricultural Research Service, Beltsville Agricultural Research Center, Building 1001, Beltsville, MD 20705, USA

Received 4 July 2004; accepted 15 July 2004

Abstract

Attempts were made to isolate *Neospora caninum* from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil. Brains from six buffaloes with indirect fluorescent antibodies (>1:100) to *N. caninum* were used to isolate the parasite by bioassay in dogs and gerbils followed by in vitro culture. Shedding of *Neospora*-like oocysts was noticed in dogs fed brains from three buffaloes (isolate designation NcBrBuf-1, 2 and 4). Two more isolates (NcBrBuf-3 and 5) were obtained by in vitro culture of the brains of gerbils previously infected with brains of two other buffaloes. The identity of the isolates was confirmed by biological and molecular methods. The isolates were found to be non-pathogenic to gerbils. All five isolates amplified the gene 5 amplicons using *Neospora*-specific PCR assay. The sequences of gene 5 fragments and the common toxoplasmiid ITS-1 fragments were analyzed. The dynamics of oocyst production in the dogs indicate

* Corresponding author. Tel.: +1 301 504 8128; fax: +1 301 504 9222.
E-mail address: jdubey@anri.barc.usda.gov (J.P. Dubey).

that water buffaloes are natural intermediate hosts for *N. caninum*. This is the first report of isolation of *N. caninum* from water buffaloes.

© 2004 Elsevier B.V. All rights reserved.

Keywords: *Neospora caninum*; Water buffaloes; *Bubalus bubalis*; Dogs; Isolation; Oocyst shedding; In vitro culture; Western blot; Sequence analysis

1. Introduction

Neospora caninum causes morbidity and mortality in dogs, cattle, horses, goats, sheep and deer (Dubey et al., 2002; Dubey, 2003a). It is one of the important causes of abortion in cattle (Dubey, 2003b). Domestic dogs and coyotes are its definitive hosts (McAllister et al., 1998; Lindsay et al., 1999; Gondim et al., 2004). Antibodies from this agent have also been reported in water buffaloes, raccoons, white-tailed deer, camels, cats and other wild canids (Dubey, 2003b). However, *N. caninum* has been isolated only from dogs, cattle and sheep (Dubey, 2003b). In this paper we report the first isolation of *N. caninum* from naturally infected water buffaloes (*Bubalus bubalis*) and confirm the identity of the isolates by biological and molecular techniques.

2. Materials and methods

2.1. Naturally infected buffaloes

Twelve 2-year-old male buffaloes from Pirassununga, São Paulo, Brazil were slaughtered in an abattoir and their sera were tested for antibodies against *N. caninum* by an indirect fluorescence antibody test (IFAT) (Fujii et al., 2001). Brains of six seropositive buffaloes (IFAT \geq 1:100, Table 1) were transported to the Laboratory of Parasite Diseases, University of São Paulo, São Paulo, Brazil, for isolation of *N. caninum*.

Table 1
Isolation of *N. caninum* from brains of seropositive buffaloes

Buffalo no.	IFAT titer	Isolation from buffalo brain				Isolate designation
		Direct in vitro cultivation ^a	Bioassay in gerbils ^a	Bioassay in dogs		
				Dog no.	Oocyst shedding	
403	100	—	—	1	+	NCBrBuf-1
406	800	+	—	6	+	NCBrBuf-2
408	200	—	—	5	—	
410	200	—	+	2	—	NCBrBuf-3
412	100	+	+	4, 7	+	NCBrBuf-4
419	200	—	+	3	—	NCBrBuf-5

^a Tachyzoites isolated from buffalo and gerbil brains were confirmed to be *N. caninum* by PCR.

2.2. Bioassay in dogs

Seven non-descript dogs, 4 of them two-months old (Nos. 1, 4, 6 and 7) and three 3-month-old (Nos. 2, 3 and 5), were dewormed, vaccinated against the common canine viral diseases and housed individually at the laboratory in São Paulo and were fed dry dog food. These dogs had never consumed raw meat or meat products prior to the study. Each dog was tested for antibodies against *N. caninum* by an IFAT (Dubey et al., 1988).

The dogs were fed part of brains from the six naturally infected buffaloes (Table 2). Feces from each dog were collected daily until day 30 or until 7 days after the last day shedding of oocysts. Each day, the total volume of feces collected in 24 h was homogenized, 5 g were mixed in 45 ml of Sheather's sugar solution, and centrifuged at $1200 \times g$ for 10 min. A drop of fluid from the upper meniscus of the solution was examined for oocysts. The number of oocysts produced was estimated from the total weight of the daily dog's fecal output. The feces containing oocysts were mixed with 2.5% potassium dichromate solution and allowed to sporulate in Petri dishes at room temperature. Sporulated oocysts were transported to the USDA laboratory at Beltsville, MD for further characterization.

Blood samples from the dogs were collected weekly until the first month and then every 2 months until 6 months, and tested for *N. caninum* antibodies by IFAT (Dubey et al., 1988). Samples with titer $> 1:50$ were considered positive for *N. caninum*.

2.3. Bioassay in Mongolian gerbils (*Meriones unguatus*)

Attempts were made to recover *N. caninum* by bioassay in gerbils. At the São Paulo laboratory, one-fourth of each buffalo brain was homogenized in an equal volume of phosphate buffer saline (PBS) with antibiotics. The suspension was filtered through sterile gauze, centrifuged at $1000 \times g$ for 10 min, and the sediment was treated with 15 ml of 0.25% trypsin solution for 30 min at 37 °C. The sediment was washed with PBS containing

Table 2
Details of oocyst shedding by dogs and confirmation of identity as *N. caninum*

Buffalo no.	Dog no.	Duration of oocyst shedding (days)	Total number of oocysts	Bioassay in gerbils and KO mice	Bioassay in cell culture	PCR of oocyst DNA ^a
403	1	26	275969	+ ^b	— ^c	+
406	6	17	820655	+	+	+
408	5	—	—	ND ^d	ND	ND
410	2	—	—	ND	ND	ND
412 ^e	4	7	21265	+	ND	+
	7	9	43500	ND	+	+
419	3	—	—	ND	ND	ND

^a As per Yamage et al. (1996).

^b Positive.

^c Negative.

^d Not determined.

^e Bioassayed in two dogs.

antibiotics by centrifugation at $1000 \times g$ for 5 min and the pellet was suspended in PBS with antibiotics (Gondim et al., 2001).

Eighteen female 8–12-week-old gerbils were obtained from Butantan Institute of São Paulo, Brazil. Three gerbils were each inoculated intraperitoneally with 2 ml of brain homogenate from each buffalo. The gerbils were euthanized 3–5 months later and their brains were used for isolation of protozoa in cell culture (see Section 2.5).

At the Beltsville laboratory, gerbils were fed sporulated oocysts obtained from feces of dogs fed buffalo brains. The gerbils were tested for antibodies to *N. caninum* and *Toxoplasma gondii* using the *Neospora* agglutination test (NAT, Romand et al., 1998) and modified agglutination test (MAT, Dubey and Desmonts, 1987), respectively, 8 weeks post-inoculation (p.i.). They were euthanized, necropsied, and their tissues were fixed in 10% neutral buffered formalin for histological and immunohistochemical evaluation.

2.4. Bioassay in interferon gamma gene knock out (KO) mice

KO mice (Dubey and Lindsay, 1998) were inoculated with brain homogenate from gerbils that were fed oocysts and with supernatant from cell culture infected with oocysts from dog No. 7. The tissues of mice that died were fixed in 10% neutral buffered formalin for histological and immunohistochemical evaluation.

2.5. Bioassay in cell culture

Attempts were made to isolate the parasite in cell cultures directly from the buffalo brain, from brain of gerbils that had been inoculated with buffalo brain and from gerbils that were fed *N. caninum*-like oocysts. Cultures were also initiated directly from the oocysts.

From each buffalo, 10 g of brain were homogenized in PBS with antibiotics and digested in trypsin as described (see Section 2.3). The digest was layered over monolayers of M 617 (bovine monocyte) cells (Dubey and Sreekumar, 2003) and maintained for 60 days in RPMI 1640 medium. Cultures were initiated similarly from the brains of infected gerbils.

Sporulated oocysts from dog Nos. 1, 6, and 7 were washed in water and concentrated as described by Sreekumar et al. (2003). Oocysts were vortexed for 5 min with 500 μm glass beads (Microbeads, Ferro Corporation, Cleveland, OH, USA) and incubated in an excystation medium [sodium taurocholate 2.5 g, sodium deoxycholic acid 4 g, trypsin (1:250) 250 mg in 100 ml saline, pH adjusted to 7.5] for 60 min at 37 °C. After the satisfactory excystation, the excystation medium was removed by centrifugation and pellet was washed once with RPMI 1640. The washed pellet was resuspended in growth medium and added to monolayers of CV-1 cells (Dubey and Sreekumar, 2003) grown on 12 mm coverslips in multi well plates.

The cultures were observed for protozoal growth. Coverslips from each isolate were removed between days 6 and 13 p.i. They were fixed in Bouin's and stained with Giemsa. The last coverslip from each isolate was stained immunohistochemically with BAG-1 and *N. caninum* antibodies (see Section 2.6).

2.6. Histology and immunohistochemistry

Paraffin-embedded sections were cut at 5 μm thickness, stained with hematoxylin and eosin (H&E), and examined microscopically. Deparaffinized sections of some tissues were stained with antibodies to *T. gondii* and *N. caninum* (Lindsay and Dubey, 1989) using procedures described by Dubey et al. (2001). Selected sections were also stained with bradyzoite-specific anti-BAG-1 antibodies as described by McAllister et al. (1996); this rabbit serum does not stain tachyzoites of *N. caninum* or *T. gondii* but stains bradyzoites of both species.

Coverslips removed from multi-well plates were fixed in acetone and stained immunohistochemically using *N. caninum* and BAG-1 antibodies (Dubey and Sreekumar, 2003).

2.7. Western blot

Antigens were prepared from the tachyzoites of the *N. caninum* isolate from dog No. 7, cultivated in CV-1 cells (ATCC CCL-70), NC-1 strain of *N. caninum* (Dubey et al., 1988, 2002), cultivated in HCT-8 cells (ATCC CCL-244), and the host cells CV-1 and HCT-8. The tachyzoites were harvested from culture supernatant and filtered through 5 μm polycarbonate membranes (Millipore Corporation, Bedford, MA, USA). Host cells were collected from a confluent monolayer, washed in PBS, and pelleted by centrifugation. The tachyzoites and cell pellets were subjected to two freeze-thaw cycles and sonicated at 20 A in 3–5 s pulses. Protein was extracted from the sonicate by the addition of reducing PAGE sample buffer (2 mM 2-mercaptoethanol, 1% SDS, 50% glycerol, pH 8.0) and boiling for 2 min. The samples were centrifuged, the supernatant was recovered, and the protein content was estimated using a modified Bradford protein assay (BioRad, Hercules, CA, USA). The same quantity of protein was used for all blots. Samples were electrophoresed on a 4–12% NuPage Bis–Tris gradient gel (Invitrogen, Carlsbad, CA, USA) in 50 mM pH 8.0 MOPS buffer at 200 V along with a marker. For Western blotting, the samples were run as above with Benchmark pre-stained ladder (Invitrogen), electroblotted onto an Immobilon-P^{sq} PVDF membrane (Millipore Corporation, Bedford, MA, USA), blocked in detector block (Kirkegaard and Perry, Gaithersburg, MD, USA), and lanes were incubated overnight in positive (from a clinical case of neosporosis) and negative control sera. Horseradish peroxidase-conjugated goat anti-dog IgG was used as the secondary antibody, and bands were visualized using 4-chloro-1-naphthol and peroxidase as the substrate (Kirkegaard and Perry, Gaithersburg, MD, USA). The lanes were analyzed and molecular weights were calculated using Labworks Image Acquisition and Analyses Software.

2.8. Polymerase chain reaction and DNA sequencing

DNA was isolated and amplified from the brains of buffaloes and from tachyzoites isolated from cell culture with buffalo (third passage) and gerbil (second passage) brains in M 617 cells. The DNA was extracted from the homogenized suspensions using DNAzol (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions.

For obtaining DNA from sporulated oocysts, suspensions were washed by repeated centrifugation in distilled water to remove the potassium dichromate. Each pellet was

treated with 10 ml of 5.25% sodium hypochlorite (on ice) to remove organic debris and then washed in water. The oocysts were ruptured by two to three freeze-thaw cycles. DNA was extracted from the suspensions using DNAeasy[®] kit (Qiagen, MD, USA) according to the manufacturer's instructions.

The extracted DNA samples were amplified with primer pairs Np6–Np21, amplifying the gene 5 region (Yamaga et al., 1996) and CT1–CT2 for common toxoplasmatid ITS-1 region (Sreekumar et al., 2003) of *N. caninum*, as previously described. Positive (*N. caninum*, NC-1 strain) and negative (water) controls were included in each test. The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide, and documented using ProExpress Gel Documentation system (Perkin Elmer, Wellesley, MA, USA).

The gel cleaned PCR products were directly sequenced in the forward and reverse directions using the Big Dye terminator system, Version 3.1 (Applied Biosystems, Foster City, CA, USA) using an ABI 377 sequencer. The sequence chromatograms were edited using Sequencher 4.1 software (Genecodes Corporation, Ann Arbor, MI, USA). BLAST searches were performed to compare the sequences with those in the public database.

3. Results

N. caninum was isolated from five of the six seropositive buffaloes (Table 1). Four of the seven dogs (Nos. 1, 4, 6 and 7) fed brains from seropositive buffaloes (Nos. 403, 406, 412 and 412) shed *N. caninum*-like oocysts. The duration of shedding and total number of oocysts shed are shown in Table 2. Two of the dogs (Nos. 1 and 4) developed IFAT titers of 1:800 or more, 2 months after ingesting buffalo brain. From two buffaloes (Nos. 406 and 412), the parasite was isolated directly by bioassay of buffalo brains in cell culture.

All 18 gerbils inoculated with buffalo's brain remained asymptomatic. However, brains from three of the gerbils, previously injected with brains from three buffaloes (Nos. 410, 412 and 419), succeeded in setting up infection of monolayers, as evidenced by the presence of tachyzoites. The isolates from buffaloes No. 403, 406, 410, 412 and 419 were designated as NCBBrBuf-1, 2, 3, 4 and 5, respectively.

All gerbils fed sporulated oocysts from dog Nos. 1, 6 and 7 also remained asymptomatic. Antibodies to *N. caninum* (NAT titers of 1:200 or more) were found in the sera of these gerbils 39 days p.i. However, *N. caninum* was not found histologically in the tissues of gerbils killed 70 days p.i. Feeding of 10-fold serial dilution of oocysts from dog No. 7 indicated that there were at least 1000 infective oocysts in the inoculum given to gerbils, seroconversion was noticed in gerbils fed a 10^{-3} dilution. Tissues from all the gerbils were negative by immunohistochemistry using *N. caninum* polyclonal rabbit antibodies.

The KO mice inoculated with gerbil tissues remained asymptomatic and did not develop antibodies to *N. caninum*. All KO mice inoculated with in vitro grown tachyzoites from dog No. 7 died of acute neosporosis. Tachyzoites were noticed in the lungs of all dead mice and could be stained by anti-*N. caninum* antibodies.

Cultures infected with brains of buffaloes No. 406 and 412 resulted in infection of monolayer and tachyzoites could be observed by 45 days p.i. Tachyzoites were seen in Giemsa stained coverslips from cultures infected with the excysted sporozoites from dog

Nos. 6 and 7. In cultures infected with oocysts of dog No. 7, tachyzoites were first noticed on day 6 p.i. By day 13 p.i., large groups of protozoa could be noticed in the cytoplasm of some infected cells. The monolayer was completely destroyed in 34 days. The parasites in culture from dog No. 7 could be stained with anti-*N. caninum* antibodies. Tachyzoites from culture were infective to gerbils and KO mice (Table 2).

The protein profile of isolate NCBBrBuf-4 (from dog No. 7) was similar, but not identical, to that of NC-1 strain. Numerous bands were shared by the two isolates. A unique band of molecular weight 56.5 kDa was seen in the profile of the Brazilian isolate, which was

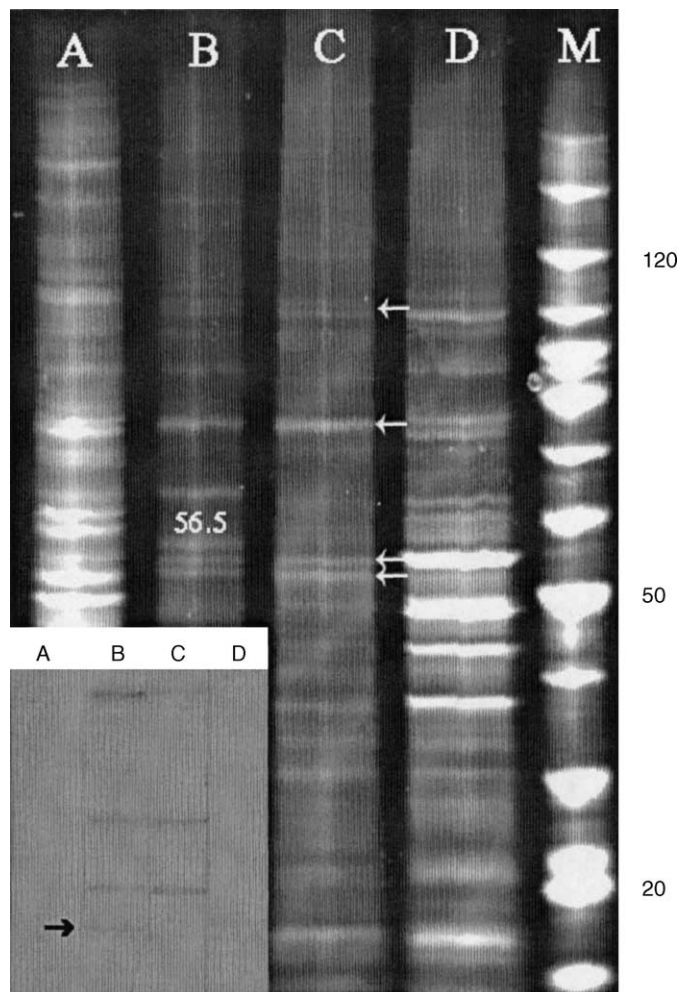


Fig. 1. Comparison of protein profiles of tachyzoite lysates of *N. caninum* isolate (NCBrBuf-4) from dog No. 7 (B) and NC-1 strain (C). Lysates of CV-1 (A) and HCT-8 (D) host cells are included as controls. Note the presence of numerous bands common between the two isolates (arrows) and a 56.5 kDa band unique to the profile of the Brazilian isolate. M = Benchmark™ Protein Ladder. Inset shows western blot with a unique band of lower molecular weight in lane B (arrow).

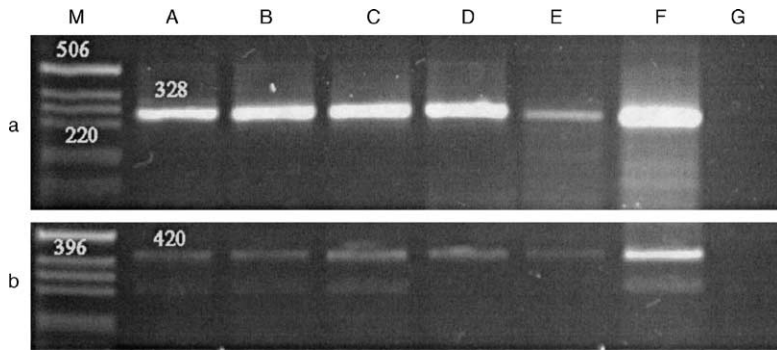


Fig. 2. Amplification of *N. caninum*-specific Gene 5 (a) and common toxoplasmatid ITS-1 (b) fragments of the isolates from buffalo No. 403 (A), 406 (B), 410 (C), 412 (D), and 419 (E) along with positive control (F, NC-1 strain) and negative control (G, water). M = 1 kb DNA ladder.

absent in NC-1 (Fig. 1). Western blot revealed three bands that were common to both isolates, with an additional band of lower molecular weight differentiating NCBBrBuf-4 from NC-1 (Fig. 1).

The identity of all isolates was confirmed as *N. caninum* by PCR. The target fragment of 328 base pairs could be amplified from DNA extracted from cell culture derived tachyzoites from brains of two buffaloes (Nos. 406 and 412), brains of gerbils infected with that of buffalo Nos. 410, 412 and 419 (Table 1), and also from oocysts shed by dogs No. 1, 4, 6 and 7 (Table 2). This band co-migrated with the positive control, and no band was amplified from negative control (Fig. 2a). All the samples were also successful in amplifying the region of ITS-1 (420 bp) common to toxoplasmatids (Fig. 2b).

The ITS-1 and gene 5 PCR products from all five isolates were sequenced. The sequences of the gene 5 region amplified from isolates NCBBrBuf-1 (AY497041), NCBBrBuf-2 (AY497042), NCBBrBuf-3 (AY497043), NCBBrBuf-4 (AY497044) and NCBBrBuf-5 (AY497045) were compared with the existing homologues in the public database. No polymorphism was noticed in the gene 5 sequences within these five Brazilian isolates. However the sequences differed at 6 nt from those of the NC-1 sequence in the public database. The ITS-1 sequences of the five Brazilian isolates (AY618482, AY618483, AY618484, AY618485, and AY618486) were also identical. The sequences were homologous to those of 17 other isolates and differed from five other isolates found in the public database. The sequences differed at 2 nt from those of the NC-1 strain (U16160) and at 7 nt from those of NC-Liverpool (L49389).

Neither *T. gondii* parasites nor antibodies to *T. gondii* (MAT, 1:25) were found in mice and gerbils inoculated with cell culture or tissues infected with the buffalo isolates of *N. caninum*.

4. Discussion

This is the first report of isolation of *N. caninum* from naturally infected adult buffaloes. There are numerous reports on successful isolation of viable *N. caninum* from cattle, from

Australia, Italy, Japan, Korea, Portugal, Sweden, UK, USA (Dubey, 2003b) and Brazil (Locatelli-Dittrich et al., 2003). The isolation of *N. caninum* from the brain of a naturally infected adult cow was reported only once by Sawada et al. (2000). This cow had previously aborted an *N. caninum* infected fetus. All the other isolates are from fetuses or calves. To our knowledge, this is the first report of isolation of *N. caninum* from any asymptomatic host.

Neospora-like tissues cysts and lesions were reported by Guarino et al. (2000) in two of the four fetuses aborted by two water buffaloes in Italy, but no tests were done to confirm the presence of *N. caninum* in these fetuses. In the absence of any published photographs, it cannot be ascertained if these fetuses had *N. caninum* infection.

In this report *N. caninum* tachyzoites were isolated in cell culture from four buffaloes. Two of them (Nos. 406 and 412) were isolated by direct culture from buffalo's brain and the other two (Nos. 410 and 419) from culture of brains of gerbils, previously inoculated with brains from seropositive buffaloes.

Gerbils have previously been shown to be susceptible to infection with tachyzoites (Cuddon et al., 1992; Gondim et al., 1999, 2001) and oocysts (Basso et al., 2001; Dubey and Lindsay, 2000) of *N. caninum*. The present study confirms the usefulness of gerbils as laboratory animals for primary isolation of *N. caninum* from naturally infected animals. Gerbils, however, are an unreliable model for continuous passage of *N. caninum*. In earlier studies, though numerous tachyzoites were seen in gerbils inoculated intraperitoneally with canine tissues infected with *N. caninum*, the parasite disappeared in the second passage (Cuddon et al., 1992). The NC-Liverpool and NC-1 strains of *N. caninum* were virulent to gerbils (Dubey and Lindsay, 2000), whereas all five isolates of *N. caninum* from buffaloes in this study were not.

In this report, the number of oocysts produced by dogs that ingested tissues from buffaloes was high. Gondim et al. (2002) reported that the number of oocysts produced by dogs that ingested tissues from infected calves was significantly higher than the number of oocysts produced by dogs that ingested infected mouse carcasses. Other reports (McAllister et al., 1998; Lindsay et al., 1999) also show a lower rate of oocyst excretion, when dogs were fed with infected mouse tissues. The higher rate of oocyst excretion by dogs that ingested brains of buffaloes infected with *N. caninum* in this study could indicate that buffaloes are natural intermediate hosts for *N. caninum*.

McAllister et al. (1998) showed that the earliest time that oocysts were produced was 8 days after ingestion of infected tissue and those are excreted for 7–19 days. Lindsay et al. (1999) observed that oocysts were produced between days 6 and 9 after ingestion. It has also been reported that dogs shed oocysts 5 days after ingestion of calf tissues, and the patency varied between 1 and 14 days (Gondim et al., 2002). The results of the present study corroborate the previous findings. McGarry et al. (2003) reported recurrence of oocyst shedding by a dog, 4 months after the first episode.

The effect of factors like age, breed, and sex of dogs on *N. caninum* oocysts excretion is largely unknown. In this report, all younger dogs shed oocysts, while older ones did not. Two of the older dogs, which did not shed oocysts, consumed buffalo brains, which were subsequently proven to be infected with *N. caninum* by bioassay in gerbils and in vitro isolation. All dogs remained clinically healthy during the experiment and two of four dogs that shed oocysts did not develop detectable antibodies for 6 months after infection. These

findings are consistent with previous studies (McAllister et al., 1998; Lindsay et al., 1999; Dijkstra et al., 2001; Schares et al., 2001; Gondim et al., 2002) on seroconversion of infected dogs.

In this study, excysted sporozoites from oocysts from two dogs were inoculated directly in cell culture and maintained as tachyzoites. One of them was confirmed to be *N. caninum* by immunohistochemistry. *N. caninum* has been cultivated in vitro in different cell lines. It can be maintained continuously in cell culture by sub-inoculation of new cultures. The NC-1 isolate of *N. caninum* isolated in 1988 has been cultivated in this way for 15 years (Dubey et al., 2002).

The protein profiles, recognized by anti-*N. caninum* sera, of tachyzoite lysates from dog No. 7 and NC-1 were similar but not identical. A 56.5 kDa band was unique to the profile of the Brazilian isolate. These results concur with those of Atkinson et al. (1999) who compared NC-Liverpool and NC-SweB1 isolates of *N. caninum* and observed similar protein profiles, with an additional band in the region of 50 kDa in NC-Liverpool. Miller et al. (2002) observed a similar but not identical antigen profile when comparing NC-Nowra and NC-Liverpool. Schock et al. (2001) did not find any significant difference between the antigenic profiles of NC-1, NC-Liverpool, BPA-1, NC-SweB1, JPA-2 and NC-LivB1 isolates by western blotting.

The identity of the isolates was confirmed by PCR, using the primers Np6/Np21 (Yamage et al., 1996). This assay does not amplify DNA of *N. hughesi* (Spencer et al., 2000), *T. gondii* (Gondim et al., 2001), or *H. heydorni* (Hill et al., 2001). PCR is considered a highly specific and sensitive technique. However, PCR assays from DNA extracted directly from the brains of seropositive buffaloes were negative, though *N. caninum* could be isolated from five of them. The negative results could be explained to be due to the lack of sensitivity resulting from the low number and sporadic distribution of parasites in the brain. PCR analyses performed on different portions from the same tissue were not always positive, indicating that parasites are not uniformly distributed in tissues (De Marez et al., 1999). Ho et al. (1997) showed that PCR amplification products from *N. caninum* infected bovine tissues were often detected in only one of the three identical tissue samples from each cow. The same low sensitivity was also observed by Pereira-Bueno et al. (2003).

The sequences for the gene 5 (Np6/Np21) fragments and ITS-1 regions obtained from all the isolates in the present study were identical to each other and to most other sequences in the database. Thus, it appears that there is very limited genetic diversity among isolates of *N. caninum*, despite there being many phenotypic differences, like virulence to gerbils and oocyst production. This apparent genetic homogeneity among isolates of *N. caninum* contrasts with the greater heterogeneity observed in the related parasite, *H. heydorni* (Sreekumar et al., in press), and can be an additional distinguishing feature between the two.

Acknowledgements

The authors thank São Paulo State Foundation for Research Support for supporting parts of this study and for the award of a fellowship to the senior author. Thanks are also due to Valsin Fournet and O.C.H. Kwok, USDA, for technical assistance.

References

- Atkinson, R., Harper, P.A., Ryce, C., Morrison, D.A., Ellis, J.T., 1999. Comparison of the biological characteristics of two isolates of *Neospora caninum*. *Parasitology* 118, 363–370.
- Basso, W., Venturini, M.C., Venturini, D.E., Hill, D.E., Kwok, O.C.H., Shen, S.K., Dubey, J.P., 2001. First isolation of *Neospora caninum* from the feces of a naturally infected dog. *J. Parasitol.* 87, 612–618.
- Cuddon, P., Lin, D.S., Bowman, D.D., Lindsay, D.S., Miller, T.K., Duncan, I.D., deLahunta, A., Cummings, J., Suter, M., Cooper, B., King, J.M., Dubey, J.P., 1992. *Neospora caninum* infection in English Springer Spaniel littermates: diagnostic evaluation and organism isolation. *J. Vet. Int. Med.* 6, 325–332.
- De Marez, T., Liddel, S., Dubey, J.P., Jenkins, M.C., Gasbarre, L., 1999. Oral infection of calves with *N. caninum* oocysts from dogs: humoral and cellular immune responses. *Int. J. Parasitol.* 29, 1647–1657.
- Dijkstra, T., Eysker, M., Schares, G., Conraths, F.J., Wouda, W., Barkena, H.W., 2001. Dogs shed *Neospora caninum* oocysts after ingestion of naturally infected bovine placenta but not after ingestion of colostrums spiked with *Neospora caninum* tachyzoites. *Int. J. Parasitol.* 31, 747–752.
- Dubey, J.P., 2003a. Neosporosis in cattle. *J. Parasitol.* 89 (Suppl.), S42–S56.
- Dubey, J.P., 2003b. Review of *Neospora caninum* and neosporosis in animals. *Korean J. Parasitol.* 41, 1–16.
- Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. *Equine Vet. J.* 19, 337–339.
- Dubey, J.P., Lindsay, D.S., 1998. Isolation in immunodeficient mice of *Sarcocystis neurona* from opossum (*Didelphis virginiana*) faeces, and its differentiation from *Sarcocystis falcatula*. *Int. J. Parasitol.* 28, 1823–1828.
- Dubey, J.P., Lindsay, D.S., 2000. Gerbils (*Meriones unguiculatus*) are highly susceptible to oral infection with *Neospora caninum* oocysts. *Parasitol. Res.* 86, 165–168.
- Dubey, J.P., Sreekumar, C., 2003. Redescription of *Hammondia hammondi* and its differentiation from *Toxoplasma gondii*. *Int. J. Parasitol.* 33, 1437–1453.
- Dubey, J.P., Hattel, W.J., Lindsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent experimental transmission. *J. Am. Vet. Med. Assoc.* 193, 1259–1263.
- Dubey, J.P., Garner, M.M., Willette, M.M., Batey, K.L., Gardiner, C.H., 2001. Disseminated toxoplasmosis in magpie geese (*Anseranas semipalmata*) with large number of tissue cysts in livers. *J. Parasitol.* 87, 219–223.
- Dubey, J.P., Barr, B.C., Barta, J.R., Bjerkas, I., Bjorkman, C., Blagburn, B.L., Bowman, D.D., Buxton, D., Ellis, J.T., Gottsteins, B., Hemphill, A., Hill, D.E., Howe, D.K., Jenkins, M.C., Kobayashi, Y., Koudela, B., Marsh, A.E., Mattsson, J.G., McAllister, M.M., Modry, D., Omata, Y., Sibley, L.D., Speer, C.A., Trees, A.J., Uggl, A., Upton, S.J., Williams, D.J.L., Lindsay, D.S., 2002. Redescription of *Neospora caninum* and its differentiation from related coccidian. *Int. J. Parasitol.* 32, 929–946.
- Fujii, T.U., Kasai, N., Nishi, S.M., Dubey, J.P., Gennari, S.M., 2001. Seroprevalence of *Neospora caninum* in female water buffaloes (*Bubalus bubalis*) from the southeastern region of Brazil. *Vet. Parasitol.* 99, 331–334.
- Gondim, L.F.P., Saeki, H., Onaga, H., Haritani, M., Yamane, I., 1999. Maintenance of *Neospora caninum* tachyzoites using Mongolian gerbils (*Meriones unguiculatus*). *NZ Vet. J.* 47, 36.
- Gondim, L.F.P., Pinheiro, A.M., Santos, P.O.M., Jesus, E.E.V., Ribeiro, M.B., Fernandes, H.S., Almeida, M.A.O., Freire, S.M., Meyer, R., McAllister, M.M., 2001. Isolation of *Neospora caninum* from the brain of a naturally infected dog, and production of encysted bradyzoites in gerbils. *Vet. Parasitol.* 101, 1–7.
- Gondim, L.F.P., Gao, L., McAllister, M.M., 2002. Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and in vitro isolation from oocysts. *J. Parasitol.* 88, 1159–1163.
- Gondim, L.F.P., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 34, 159–161.
- Guarino, A., Fusco, G., Savini, G., Francesco, G.D.I., Crincoli, G., 2000. Neosporosis in water buffalo (*Bubalus bubalis*) in southern Italy. *Vet. Parasitol.* 91, 15–21.
- Hill, D.E., Liddel, S., Jenkins, M.C., Dubey, J.P., 2001. Specific detection of *Neospora caninum* oocysts in fecal samples from experimentally-infected dogs using the polymerase chain reaction. *J. Parasitol.* 87, 395–398.
- Ho, M.S.Y., Barr, B.C., Rowe, J.D., Anderson, M.L., Sverlow, K.W., Packham, A., Marsh, A.E., Conrad, P.A., 1997. Detection of *Neospora sp.* from infected bovine tissues by PCR and probe hybridization. *J. Parasitol.* 83, 508–514.

- Lindsay, D.S., Dubey, J.P., 1989. Immunohistochemical diagnosis of *Neospora caninum* in tissues sections. Am. J. Vet. Res. 50, 1981–1983.
- Lindsay, D.S., Dubey, J.P., Duncan, R.B., 1999. Confirmation that the dog is a definitive host for *Neospora caninum*. Vet. Parasitol. 82, 327–333.
- Locatelli-Dittrich, R., Richartz, R.R.T.B., Gasino-Joineau, M.E., Pinckney, R.D., Sousa, R.S., Leite, L.C., Thomaz-Soccol, V., 2003. Isolation of *Neospora caninum* from a blind calf in Parana, southern Brazil. Vet. Rec. 20, 366–367.
- McAllister, M.M., Parmley, S.F., Weiss, L.M., Welch, V.J., McGuire, A.M., 1996. An Immunohistochemical method for detecting bradyzoites antigen (BAG-5) in *Toxoplasma gondii*-infected tissues cross reacts with a *Neospora caninum* bradyzoites antigen. J. Parasitol. 82, 354–355.
- McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 28, 1473–1478.
- McGarry, J.W., Stockton, C.M., Williams, D.J.L., Trees, A.J., 2003. Protracted shedding of oocysts of *Neospora caninum* by naturally infected foxhound. J. Parasitol. 89, 628–630.
- Miller, C.M.D., Quinn, H.E., Windsor, P.A., Ellis, J.T., 2002. Characterization of the first Australian isolate of *Neospora caninum* from cattle. Aust. Vet. J. 80, 620–625.
- Pereira-Bueno, J., Quintanilla-Gonzalo, A., Perez-Perez, V., Espi-Felgueroso, A., Alvarez-Garcia, G., Collantes-Fernandez, E., Ortega-Mora, L.M., 2003. Evaluation by different diagnostic techniques of bovine abortion associated with *Neospora caninum* in Spain. Vet. Parasitol. 111, 413–452.
- Romand, S., Thulliez, P., Dubey, J.P., 1998. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol. Res. 84, 50–53.
- Sawada, M., Kondo, H., Tomioko, Y., Park, C.H., Morita, T., Shimada, A., Umemura, T., 2000. Isolation of *Neospora caninum* from the brain of a naturally infected adult dairy cow. Vet. Parasitol. 90, 247–252.
- Schares, G., Heydorn, A.O., Cuppers, A., Conraths, F.J., Mehlhorn, H., 2001. *Hammondia heydorni*-like oocysts shed by a naturally infected dog and *Neospora caninum* NC-1 cannot be distinguished. Parasitol. Res. 87, 808–816.
- Schock, A., Innes, E.A., Yamane, I., Lathan, S.M., Wastling, J.M., 2001. Genetic and biological diversity among isolates of *Neospora caninum*. Parasitology 123, 13–23.
- Spencer, J.A., Withrow, A.K., Blagburn, B.L., 2000. A random amplified polymorphic DNA polymerase chain reaction technique that differentiates between *Neospora* species. J. Parasitol. 86, 1366–1368.
- Sreekumar, C., Hill, D.E., Fournet, V.M., Rosenthal, B.M., Lindsay, D.S., Dubey, J.P., 2003. Detection of *Hammondia heydorni*-like organisms and their differentiation from *Neospora caninum* using random-amplified polymorphic DNA-polymerase chain reaction. J. Parasitol. 89, 1082–1085.
- Sreekumar, C., Hill, D.E., Miska, K.B., Rosenthal, B.M., Vianna, M.C.B., Venturini, L., Basso, W., Gennari, S.M., Lindsay, D.S., Dubey, J.P., in press. *Hammondia heydorni*: evidence of genetic diversity among isolates from dogs. Exp. Parasitol. 107, 65–71.
- Yamage, M., Flechtner, O., Gottstein, B., 1996. *Neospora caninum*: specific oligonucleotide primers for the detection of brain “cyst” DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). J. Parasitol. 82, 272–279.